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## Determinants of the VP1/2A junction cleavage by the 3C protease in foot-and-mouth disease virus infected cells --Manuscript Draft--

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<b>Abstract:</b>	<p>The foot-and-mouth disease virus (FMDV) capsid precursor, P1-2A, is cleaved by FMDV 3C protease to yield VP0, VP3, VP1 and 2A. Cleavage of the VP1/2A junction is the slowest. Serotype O FMDVs with uncleaved VP1-2A (having a K210E substitution in VP1; at position P2 in cleavage site) have been described previously and acquired a second site substitution (VP1 E83K) during virus rescue. Furthermore, introduction of the VP1 E83K substitution alone generated a second site change at the VP1/2A junction (2A L2P, position P2' in cleavage site). These virus adaptations have now been analysed using Next Generation Sequencing to determine sub-consensus level changes in the virus; this revealed other variants within the E83K mutant virus population that changed residue VP1 K210. The construction of serotype A viruses with a blocked VP1/2A cleavage site (containing K210E) has now been achieved. A collection of alternative amino acid substitutions were made at this site and the properties of the mutant viruses determined. Only the presence of a positively charged residue at position P2 in the cleavage site permitted efficient cleavage of the VP1/2A junction, consistent with analyses of diverse FMDV genome sequences. Interestingly, in contrast to the serotype O virus results, no second site mutations occurred within the VP1 coding region of serotype A viruses with the blocked VP1/2A cleavage site. However, some of these viruses acquired changes in the 2C protein that is involved in enterovirus morphogenesis. These results have implications for the testing of potential antiviral agents targeting the FMDV 3C protease.</p>

**Determinants of the VP1/2A junction cleavage by the 3C protease in foot-and-mouth disease virus infected cells**

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## 26    **Abstract**

27    The foot-and-mouth disease virus (FMDV) capsid precursor, P1-2A, is cleaved by FMDV 3C  
28    protease to yield VP0, VP3, VP1 and 2A. Cleavage of the VP1/2A junction is the slowest. Serotype  
29    O FMDVs with uncleaved VP1-2A (having a K210E substitution in VP1; at position P2 in cleavage  
30    site) have been described previously and acquired a second site substitution (VP1 E83K) during virus  
31    rescue. Furthermore, introduction of the VP1 E83K substitution alone generated a second site change  
32    at the VP1/2A junction (2A L2P, position P2' in cleavage site). These virus adaptations have now  
33    been analysed using Next Generation Sequencing to determine sub-consensus level changes in the  
34    virus; this revealed other variants within the E83K mutant virus population that changed residue VP1  
35    K210. The construction of serotype A viruses with a blocked VP1/2A cleavage site (containing  
36    K210E) has now been achieved. A collection of alternative amino acid substitutions were made at  
37    this site and the properties of the mutant viruses determined. Only the presence of a positively charged  
38    residue at position P2 in the cleavage site permitted efficient cleavage of the VP1/2A junction,  
39    consistent with analyses of diverse FMDV genome sequences. Interestingly, in contrast to the  
40    serotype O virus results, no second site mutations occurred within the VP1 coding region of serotype  
41    A viruses with the blocked VP1/2A cleavage site. However, some of these viruses acquired changes  
42    in the 2C protein that is involved in enterovirus morphogenesis. These results have implications for  
43    the testing of potential antiviral agents targeting the FMDV 3C protease.

44

## 45    **Introduction**

46    Foot-and-mouth disease virus (FMDV) is the prototypic member of the *Aphthovirus* genus within the  
47    family *Picornaviridae* and seven different serotypes (O, A, C, SAT 1-3 and Asia-1) have been  
48    identified. All FMDVs have a positive sense RNA genome (ca. 8400 nt) that includes a single large  
49    open reading frame (ca. 7000 nt) encoding a polyprotein (Belsham, 2005). The full-length polyprotein

is never observed since during, and after, synthesis it is processed, mainly by virus-encoded proteases, to generate 15 distinct mature products plus multiple precursors. The FMDV polyprotein includes two *trans*-acting proteases; these are the Leader (L) protease and the 3C protease (3C<sup>pro</sup>). The L protease is only responsible for one cleavage within the polyprotein that occurs at its own C-terminus (i.e. the L/P1-2A junction, Strebel & Beck, 1986; Medina *et al.*, 1993). However, this protease also induces cleavage of the translation initiation factor eIF4G, this results in the inhibition of host cell, cap-dependent, protein synthesis (reviewed in Belsham, 2005). The 3C<sup>pro</sup> cleaves all the other junctions within the FMDV polyprotein except for the VP4/VP2 junction and the 2A/2B junction. Cleavage of VP0 to VP4 and VP2 occurs on encapsidation of the viral RNA and also within assembled empty capsid particles (Curry *et al.*, 1995; Gullberg *et al.*, 2013a; Gullberg *et al.*, 2013b). Separation of the 2A peptide from the 2B protein is dependent on the 2A coding sequence. However, this region only encodes 18 amino acids (without any protease motifs) but its presence results in a break in the polyprotein during its synthesis; this is described as “ribosomal skipping” (Donnelly *et al.*, 2001) or “StopGo” (Atkins *et al.*, 2007).

The FMDV capsid protein precursor, P1-2A (Fig. 1), is processed by the 3C<sup>pro</sup> to VP0, VP3, VP1 and 2A. The scission of the VP1-2A junction is the slowest of these cleavages within cell-free translation systems (Ryan *et al.*, 1989) and within mammalian cells (Gullberg *et al.*, 2013a; Gullberg *et al.*, 2013b) since the uncleaved VP1-2A can still be detected when all other junctions are fully cleaved (e.g. when P1-2A is expressed with a low-level of 3C<sup>pro</sup>). However, in peptide cleavage assays, using short synthetic substrates, it has been found that the peptide corresponding to the VP1/2A cleavage site was the most rapidly processed (Birtley *et al.*, 2005).

The FMDV 3C<sup>pro</sup> cleaves a variety of different junction sequences (the amino acid residues at the cleavage junctions are indicated as: P4P3P2P1/P1'P2'P3'P4'). The cleavage sites recognized by the FMDV 3C<sup>pro</sup> have either glutamine (Gln, Q) or glutamate (Glu, E) at the P1 position (Curry *et al.*,

2007). The consensus sequence (in single letter code) for the VP1/2A junction in serotype O and A FMDVs is PxKQ/xLNF. The Q residue at the P1 position together with the P4-Pro (P), P2-Lys (K) and P4'-Phe (F) residues, represent key determinants of 3C<sup>pro</sup> specificity at this site (Birtley *et al.*, 2005; Curry *et al.*, 2007; Zunszain *et al.*, 2010). Analysis of aligned FMDV 3C<sup>pro</sup> cleavage sites from over 100 strains of the virus (including representatives of all serotypes) revealed that sites with P1-Q have a strong selectivity for P2-K, indicating that recognition of the P1 residue by 3C<sup>pro</sup> is influenced by the P2 residue (Curry *et al.*, 2007). Recently, we have shown that changing the P2-K residue to E at the VP1/2A junction (i.e. K210E in VP1), in a serotype O virus, greatly inhibited cleavage at this junction and resulted in the formation of infectious virus particles containing the uncleaved VP1-2A (Gullberg *et al.*, 2013b). The “self-tagged” viruses containing this modification (K210E) also acquired, during the virus rescue procedure, a second amino acid substitution within VP1 (E83K). Interestingly, introduction of this E83K substitution alone into the virus, generated a second site mutant (L2P, in the 2A sequence; this is position P2' in the VP1/2A junction) that also blocked cleavage (Gullberg *et al.*, 2014). We have now expanded this analysis to identify the determinants of cleavage at the VP1/2A junction within the context of infectious serotype O and A FMD viruses using a variety of different approaches. Within the serotype A FMDVs, no second site changes in VP1 were observed in viruses where the VP1/2A cleavage was inhibited, but some evidence for changes in 2C was obtained. For certain picornaviruses, within the enterovirus genus, interactions between the virus capsid proteins and the 2C non-structural protein have been implicated in the process of virus morphogenesis (Liu *et al.*, 2010; Wang *et al.*, 2012; Wang *et al.*, 2014).

94

## 95 **Results**

### 96 *Modification of the VP1/2A cleavage site in a serotype A FMDV.*

97 In order to determine whether the key elements of the results obtained with the serotype O FMDV  
98 sequences (Gullberg *et al.*, 2014) also applied to serotype A FMDV, the effect of modifying the

99 VP1/2A cleavage site within a serotype A FMDV was examined. The VP1/2A cleavage site sequence  
100 in the A22 strain of FMDV (APAKQ/LLNFD) differs at just 1 out of the 10 residues flanking the  
101 junction from the serotype O (strain O1 Manisa, abbreviated throughout as O1M) sequence  
102 (APVKQ/LLNFD) analysed previously (Gullberg *et al.*, 2013b; Gullberg *et al.*, 2014) (see Fig. 1).  
103 The K210E substitution (at the P2 position) was introduced into a full-length FMDV cDNA, based  
104 on the backbone of a chimeric O1 Kaufbeuren (O1K) virus containing the coding sequence for the  
105 VP2-VP3-VP1-2A region of the A22 capsid protein precursor (Fig.1). Virus was successfully rescued  
106 from this chimeric wt O1K/A22 plasmid and also from the O1K/A22 VP1 K210E mutant (this had  
107 changed the codon encoding VP1 residue 210 from AAA to GAA). However, when the capsid protein  
108 coding sequences within the rescued virus were determined, it was found that the K210E substitution  
109 in VP1 had reverted in the virus by passage 2 (Psg 2) to the wt sequence (this only requires a single  
110 nt change) (see Fig. 1).

111 Two further modifications were, therefore, introduced into the serotype A viruses, the 2A L2P  
112 modification was made in isolation (using 3 nt changes, TTG to CCA) and a double mutant containing  
113 both the K210E substitution (the single nt change) and the 2A L2P substitution. Viruses were rescued  
114 successfully from both of these mutant plasmids. Consensus sequencing indicated that the expected  
115 mutations were still present within these rescued viruses and that no other mutations were detected  
116 within the VP2-2A coding region (see Fig. 1).

117 Analysis of the FMDV capsid proteins within cells infected with the wt and mutant O1K/A22 viruses,  
118 as determined by immunoblotting using anti-VP2 and anti-2A antibodies, is shown in Fig. 2. As  
119 expected, the production of VP0 and VP2 was very similar in each of the infected cell extracts (Fig.  
120 2(a), lanes 2-7). However, the presence of the uncleaved VP1-2A was only observed with the mutant  
121 viruses, either containing the 2A L2P substitution alone (Fig. 2(b), lanes 4 and 5) or with the double  
122 mutant (VP1 K210E and 2A L2P) (Fig. 2(b), lanes 6 and 7).

123 These results were confirmed using immunofluorescence (IF) studies (Fig. 3). Consistent with the  
124 immunoblotting data, the presence of FMDV 2A (still attached to VP1) was detected in cells infected  
125 with the O1K/A22 2A L2P mutant virus (Fig. 3(g)) and with the double mutant (O1K/A22 VP1  
126 K210E and 2A L2P) virus (Fig. 3(h)). In contrast, no signal for the 2A peptide was observed in cells  
127 infected with the wt O1K/A22 virus (Fig. 3(f)) or in uninfected cells (Fig. 3(e)). The presence of the  
128 FMDV capsid proteins could be detected in cells infected with each of the viruses (Fig. 3(b-d)) but  
129 not in uninfected cells (Fig. 3(a)). These results are consistent with those obtained using the serotype  
130 O FMDVs previously (Gullberg *et al.*, 2014). It seems that the free 2A peptide is not efficiently  
131 detected within cells using the IF approach; it is assumed that either it breaks down very quickly or  
132 the conditions of the IF assay are not suitable for detection of this short peptide.

133

#### 134 *Use of NGS to determine sequence diversity within rescued FMDVs.*

135 As described previously (Gullberg *et al.*, 2013b; Gullberg *et al.*, 2014), consensus sequencing of the  
136 capsid coding region (P1-2A) of the serotype O FMDVs rescued from the K210E and E83K mutant  
137 forms of the O1K/O1M cDNAs identified the presence of additional amino acid substitutions in the  
138 rescued viruses. We wished to analyse these adaptations in more detail, in particular to examine the  
139 appearance of sub-consensus level changes throughout the near complete genome sequence including  
140 the complete polyprotein coding region. To achieve this, RNA was extracted from the rescued  
141 O1K/O1M VP1 E83K and O1K/O1M VP1 K210E viruses (as described by Gullberg *et al.*, 2013b;  
142 Gullberg *et al.*, 2014) at Psg 2 and/or Psg 3. Two separate, but overlapping, cDNA fragments  
143 including the near complete genome (ca. 8kb, downstream of the poly(C) tract) were produced by  
144 RT-PCR, mixed and then sequenced using NGS at a coverage of about 5,000 reads per nt (except  
145 near the extreme 5'- and 3'-termini).



146 This analysis showed that the rescued virus O1K/O1M VP1 E83K retained the expected substitution  
147 (encoding E83K) in 100% of the progeny at Psg 2 but there was some heterogeneity in the sequence  
148 near the VP1/2A junction (see Table 1). As described earlier, the consensus sequencing indicated that  
149 a substitution (L2P) within the 2A sequence occurred within this rescued virus (Gullberg *et al.*, 2014).  
150 The analysis by NGS (see Table 1) demonstrated that at Psg 2 some 83% of the reads corresponded  
151 to this L2P substitution in 2A while two other variants were also present (albeit at relatively low  
152 levels, 4% and 9%) which each encoded the K210N substitution in VP1 (c.f. the K210E change  
153 described previously in O1M, (Gullberg *et al.*, 2014)). No other coding changes were present  
154 anywhere in the genome at an abundance of more than 3%.

155 Consensus sequence analysis of the rescued O1K/O1M VP1 K210E virus has shown previously the  
156 generation of the E83K substitution (Gullberg *et al.*, 2013b). Using NGS, it was found that the E83K  
157 substitution in VP1 was encoded in 78% of the reads generated at Psg 2 and in 99% of the reads at  
158 Psg 3 (Table 1), consistent with the earlier consensus sequencing (Gullberg *et al.*, 2013b). The K210E  
159 substitution was maintained in this rescued virus in 100% of the reads at Psg 2 and Psg 3.

160 For the rescued serotype A viruses, consensus (Sanger) sequencing of the rescued O1K/A22 2A L2P  
161 virus demonstrated the maintenance of the introduced changes and did not reveal any additional  
162 modifications resulting in amino acid substitutions within the P1-2A coding region. This was  
163 confirmed by NGS but an A to G nucleotide change, resulting in a single amino acid substitution  
164 (T44 to A within the 2C protein), was found in 13% of the reads from the rescued virus (Table 2).  
165 Within the O1K/A22 VP1 K210E and 2A L2P virus, only the expected changes in the P1-2A coding  
166 region were observed but an additional change, resulting in the amino acid substitution A73V in the  
167 2C protein, was detected in 100% of the sequence reads, see Table 2. The plasmid pO1K/A22 K210E  
168 L2P from which this virus was rescued has been confirmed as having the expected sequence in the  
169 2C coding region (100% identity to O1K, data not shown) and thus this sequence change had occurred

170 during virus rescue. The significance of these changes in 2C is unknown; neither residue is completely  
171 conserved among FMDV strains (Carrillo *et al.*, 2005). The A73V change in 2C was not present in  
172 the rescued O1K/A22 wt or the O1K/A22 2AL2P and there was no evidence for the T44A amino acid  
173 substitution in either the O1K/A22 wt or the O1K/A22 VP1 K210E and 2A L2P virus that was  
174 encoded in a minority of the O1K/A22 2A L2P virus reads.

175

#### 176 *Exploring potential sequence diversity at the VP1/2A cleavage site.*

177 The results described previously (Gullberg *et al.*, 2013b; Gullberg *et al.*, 2014) indicated that  
178 modification of the VP1/2A junction sequence from PxKQ/xLNF at the P2 position (from K to E) or  
179 the P2' position (from L to P) was sufficient to strongly inhibit cleavage by 3C<sup>pro</sup> at this protein  
180 junction but these changes did not affect virus viability. Furthermore, the NGS data (Table 1)  
181 indicated that the K to N substitution at residue 210 of VP1 was also probably viable since some 13%  
182 of the virus population acquired this change. To establish the range of amino acid substitutions that  
183 could be tolerated at this junction, mutagenesis of the codon for residue 210 within VP1 was  
184 undertaken within the context of the O1K/A22 full-length cDNA. The mutagenesis generated 9  
185 different codon sequences that encoded 7 distinct amino acid substitutions, see Table 3. RNA  
186 transcripts were produced from the mutant cDNAs and electroporated into BHK cells and infectious  
187 viruses were rescued in each case. From the virus harvests, RNA was extracted and the sequence of  
188 VP1-2A coding region was determined, changes (if any) are shown in Table 3. Consistent with the  
189 studies described above, the O1K/A22 VP1 K210E mutant (with a single nt change, GAA) again  
190 reverted to the parental sequence, however when 2 nt changes were introduced (GAG) to produce the  
191 K210E substitution then viruses that retained these 2 nt changes were obtained. Thus the VP1  
192 substitutions K210Q, K210R, K210A, K210V, K210M, K210N and K210E (as the GAG double  
193 mutant) were all viable without reversion or other adaptation within VP1 (Table 3). Using consensus

level sequencing, no changes in the 2C coding region were detected in any of these rescued viruses either (data not shown).

The cleavage of the VP1/2A junction in cells infected with the rescued mutant viruses was assessed using the IF assay, as described above. The presence of the FMDV capsid proteins (using anti-FMDV antisera) and of the VP1-2A (using anti-2A antibodies) was determined in BHK cells infected with the different viruses. FMDV infection, but with no staining for VP1-2A, was observed in cells infected with the viruses containing the VP1 residue K210 (wt) and R210 (Fig. 4). In contrast, cells infected with the rescued viruses having the substitutions in VP1 K210Q, K210A, K210V, K210M, K210N and K210E (double mutant) each showed staining both for the FMDV capsid proteins and for 2A ( using the anti-2A antisera), indicative of blocked VP1/2A cleavage (see Fig. 4 and Table 3). No staining with either antiserum was observed in uninfected cells, as expected (Fig. 4).

To confirm these results, immunoblotting was performed using anti-2A antibodies to determine the presence of the uncleaved VP1-2A within infected cells. The results are shown in Fig. 5(a). Consistent with the IF results, no VP1-2A product was detected in uninfected cells or in wt (K210) or mutant K210R FMDV-infected cells. In contrast, the presence of VP1-2A was readily apparent within cells infected with mutant FMDVs having the K210Q, K210A, K210V, K210M, K210N and K210E substitutions. The presence of FMDV capsid proteins in the lysates from cells infected with each of the different FMDV variants was verified using anti-VP2 antibodies that recognizes both VP0 and VP2 (Fig. 5(b)). These results support the IF data and indicate that the VP1/2A junction is only cleaved when residue 210 in VP1 is either K or R (these are both basic residues).

214

## 215 Discussion

216 In our earlier studies, it was shown that the K210E substitution in VP1 within the FMDV O1M capsid  
217 inhibited cleavage of the VP1/2A junction and resulted in generation of a second site substitution

218 (E83K in VP1) in the mutant virus (Gullberg *et al.*, 2013b). Furthermore, introduction of the E83K  
219 substitution alone in VP1 resulted in the production of another second site change (with a substitution  
220 of L2P in 2A) that also blocked VP1/2A junction processing in cells infected with the rescued virus  
221 (Gullberg *et al.*, 2014). In contrast, this study, has shown that when the K210E substitution (GAA  
222 mutant) was introduced into the VP1 of a serotype A virus (O1K/A22), the virus reverted to wt (AAA)  
223 very rapidly (single nt change). However, introduction of 2 nt changes (GAG codon) enabled the  
224 K210E substitution to be maintained. Consistent with the serotype O virus results (Gullberg *et al.*,  
225 2013b; Gullberg *et al.*, 2014), this amino acid substitution alone was sufficient to block VP1/2A  
226 cleavage (see Figs. 4 and 5). In addition, introduction of the 2A L2P change alone (employing 3 nt  
227 changes) or also with the K210E substitution resulted in the generation of viruses which maintained  
228 each of these changes. Furthermore, the presence of the uncleaved VP1-2A protein was observed  
229 within cells infected with these serotype A viruses (Figs. 2 and 3). Thus, consistent with results using  
230 the O1K/O1M FMDV (Gullberg *et al.*, 2013b; Gullberg *et al.*, 2014), it is possible to obtain infectious  
231 serotype A FMDVs, containing the uncleaved VP1-2A. However, in contrast to the results using the  
232 serotype O virus, there was no apparent selection for a substitution at residue E83 in VP1 (or  
233 elsewhere within the VP1) within the serotype A background. The basis for this difference is not  
234 known but it is noteworthy that the serotype A capsid proteins assemble into FMDV empty capsids  
235 much more efficiently than the serotype O proteins (Abrams *et al.*, 1995; Porta *et al.*, 2013). It is also  
236 apparent that some of the rescued serotype A viruses, with the VP1/2A junction rendered non-  
237 cleavable, acquired second site mutations within the non-structural protein 2C (see Table 2). In  
238 particular, the A73V variant within 2C was encoded by 100% of the sequence reads at Psg 3 of the  
239 rescued O1K/A22 VP1-K210E and 2A-L2P virus. This suggests a strong selective pressure for this  
240 amino acid substitution. The significance of this is currently unknown but there is some evidence for

241 interactions between the capsid proteins and the 2C protein of enteroviruses (e.g. poliovirus) during  
242 virus morphogenesis (Liu *et al.*, 2010; Wang *et al.*, 2012; Wang *et al.*, 2014).

243 Interestingly, it was also observed, using NGS, that during the rescue of the O1K/O1M VP1 E83K  
244 virus a minority of the virus population encoded a K210N substitution in VP1 (c.f. the K210E  
245 substitution observed in a laboratory grown O1M virus, Gullberg *et al.*, 2013b). It should be noted  
246 that the majority of the serotype O VP1 E83K mutant virus RNA acquired the 2A L2P substitution  
247 during the virus rescue procedure (see Table 1). This encouraged analysis of the range of substitutions  
248 that can be accommodated at residue 210 in VP1; this is at position P2 relative to the VP1/2A cleavage  
249 site. Within the serotype A background, there appears to be significant selection pressure against the  
250 K210E substitution, since following independent transfections of a mutant RNA containing a single  
251 nt change (GAA) reversion to the wt (AAA) sequence occurred. However, when 2 nt changes were  
252 used (GAG) to make this amino acid substitution then the K210E substitution was maintained. The  
253 single nt substitution to make the K210E substitution was also maintained in the O1K/A22 K210E  
254 and L2P double mutant; presumably the presence of the two substitutions that blocked VP1/2A  
255 cleavage overcame the selective pressure for reversion. In addition, a range of other amino acid  
256 substitutions were tolerated, most of these substitutions (e.g. K210A, K210V, K210M, K210N)  
257 blocked cleavage of the VP1/2A junction, thus the VP1-2A product was stable (see Figs. 4 and 5). In  
258 contrast, the 2A peptide was released from the VP1 in the K210R mutant. Therefore, it appears that  
259 the presence of a positively charged residue (K or R) at residue 210 in VP1 is essential for VP1/2A  
260 cleavage and a negative charge (as in K210E) is less well tolerated by itself and was selected against.  
261 These results are consistent with the strong predominance of the K and R residues at this position in  
262 the “logos plot” generated by Curry *et al.*, (2007) for 3C<sup>pro</sup> cleavage sites, with Q at the P1 position,  
263 based on the known sequences of over 100 strains of FMDV.

264 Using peptide cleavage assays, the VP1/2A peptide was the most efficiently cleaved substrate for  
265 FMDV 3C<sup>pro</sup> (Birtley *et al.*, 2005). However, making the K210A substitution abrogated cleavage of  
266 this peptide, in accordance with the results presented here. Zunszain *et al.*, (2010), have described  
267 additional modifications to the peptide substrate, which corresponds to the VP1/2A junction.  
268 Changing the P2 residue in the cleavage site (corresponding to K210 in VP1) from K to R or to  
269 ornithine (also positively charged) had relatively modest effects on the cleavage rate. However,  
270 substitution to the neutral T residue abrogated cleavage as observed here with the K210 changed to  
271 Q, A, V, M or N (see Table 3, Figs. 4 and 5).

272 It has been proposed that the FMDV 3C<sup>pro</sup> may be a good target for the development of an antiviral  
273 agent (Roqué Rosell *et al.*, 2014). Furthermore, it was shown that compounds that resemble the  
274 peptide substrate can act as an efficient inhibitor of this protease. The presence of a positively charged  
275 residue at the P2 position generated the most effective inhibitors while compounds containing neutral  
276 residues (e.g. G and Q) or a negatively charged residue (E) at this position were much less effective,  
277 consistent with the poor cleavage of the VP1/2A junction seen here in viruses containing equivalent  
278 substitutions.

279 It is important to note that viable FMDVs with the VP1/2A junction uncleaved can be obtained (as  
280 here, and as described previously for serotype O viruses, Gullberg *et al.*, 2013b; Gullberg *et al.*, 2014).  
281 Thus, it may be wise to focus screens for such antiviral agents on other 3C<sup>pro</sup> cleavage sites so that  
282 the block on polyprotein processing is most effective at inhibiting virus replication.

283 The results presented here demonstrate that the combination of reverse genetics and NGS provides  
284 powerful tools to direct and identify virus adaptation thus permitting novel aspects of the virus  
285 biology to be identified.

286

## 287 **Material and Methods**

288 *Plasmid construction*

289 The structures of plasmids containing full-length FMDV cDNAs used in this study are indicated in  
290 Fig.1. The chimeric pO1K/A22 plasmids (containing the A22 Iraq capsid coding sequences, as used  
291 in Porta *et al.*, 2013; Polacek *et al.*, 2013) in the FMDV O1 Kaufbeuren (O1K) backbone were  
292 generated using the same procedures as used previously for the production of the pO1K/O1M VP1  
293 K210E (Gullberg *et al.*, 2013b). Briefly, the cDNAs corresponding to the A22 VP2-2A coding region  
294 were amplified from pGEM-3Z-A-P1-2A-mIRES-3C (Gullberg *et al.*, 2013a) and pGEM-3Z-A-P1-  
295 2A-mIRES-3C VP1K210E (Gullberg *et al.*, 2013b) with primers FMDVA\_*NheI*VP4VP2\_Fw and  
296 FMDVA\_*ApaI*2A2B\_Re (see Supplementary Table S1) and used to generate the full-length cDNAs  
297 termed pO1K/A22 wt and pO1K/A22 VP1 K210E, respectively (Fig.1). In order to produce  
298 pO1K/A22 2A L2P and the double mutant pO1K/A22 VP1 K210E and 2A L2P, intermediate  
299 plasmids (using pO1K/A22 wt and pO1K/A22 VP1K210E, as described above, as templates) were  
300 generated. This was achieved using the QuikChange site-directed mutagenesis method (with  
301 *Pfu*Turbo DNA polymerase; Stratagene), according to the manufacturer's instructions, with primers  
302 containing the desired modifications (see Supplementary Table S1, namely FMDVA\_2AL2P\_Fw  
303 together with FMDVA\_2AL2P\_Re or FMDVA\_VP1K210E\_2AL2P\_Re). The subsequent steps to  
304 produce the four pO1K/A22 variants were performed essentially as described for the serotype O  
305 plasmid pO1K/O1M VP1 E83K (Gullberg *et al.*, 2014). Plasmids were amplified in *E. coli* Top10  
306 cells (Invitrogen), purified (Midiprep kit; Thermo Scientific) and verified by sequencing of the capsid  
307 coding region (and for pO1K/A22 VP1 K210E the 2C coding region as well) with a BigDye  
308 Terminator v. 3.1 Cycle Sequencing Kit and a 3500 Genetic Analyzer (Applied Biosystems).  
309 Additional mutations, encoding changes at the VP1/2A junction, were introduced into the pO1K/A22  
310 full-length FMDV cDNA (Fig.1) by site-directed mutagenesis using a megaprimer (146 bp) that was  
311 prepared by PCR using primer O1PN20 and primer 13LPN21, (see Supplementary Table S1), that

312 had NNN at the position corresponding to the codon for the VP1 residue 210. This degenerate  
313 megaprimer was used, with the pO1K/A22 full-length cDNA template and *Pfu*Turbo DNA  
314 polymerase (as above) to generate a collection of 9 plasmids encoding a variety of different amino  
315 acids, with diverse properties, in place of VP1 residue K210; this is at position P2 relative to the  
316 VP1/2A cleavage site. The details of each modification made are indicated in Table 3.

317

#### 318 *Rescue of modified viruses from cDNA.*

319 Plasmids pO1K/A22 (wt), pO1K/A22 VP1 210E, pO1K/A22 2A L2P and pO1K/A22 VP1 K210E  
320 2A L2P, (as shown in Fig.1) containing the full-length FMDV cDNA sequences were linearized by  
321 digestion with *HpaI*, purified (using a QIAquick PCR purification kit; Qiagen) and transcribed *in*  
322 *vitro* using T7 RNA polymerase (Megascript kit; Ambion). The transcripts were analyzed using  
323 agarose gel electrophoresis and then introduced into baby hamster kidney (BHK) cells by  
324 electroporation as described previously (Nayak *et al.*, 2006; Bøtner *et al.*, 2011). At 2 days post  
325 electroporation, the rescued viruses were harvested and amplified in one, or two, subsequent passages  
326 (Psg 2 and Psg3) in BHK cells. After these passages, viral RNA was extracted (QIAamp RNA blood  
327 mini kit; Qiagen), reverse transcribed using ready-to-go you-prime first-strand beads (GE Healthcare  
328 Life Sciences), and the FMDV cDNA corresponding to the VP2-2A coding region was amplified in  
329 a PCR (Expand high-fidelity PCR system; Roche). Control reactions, lacking reverse transcriptase,  
330 were used to show that the PCR products obtained were derived from the viral RNA and not from  
331 residual DNA template. The amplicons (~2,000 bp) including the entire VP2-2A coding region were  
332 visualized in agarose gels, purified (GeneJET gel extraction kit, Thermo Scientific) and sequenced,  
333 on both strands, as above. Sequences were analysed using Vector NTI software (Invitrogen).

334 For the library of VP1 K210 mutants, mutant viruses were rescued essentially as described above.

335 The sequencing covered the VP1-2A coding region (from a PCR product of ca. 700bp) both before



336 and after virus rescue. The sequence of the 2C coding region was also determined in selected cases.  
337 The rescued viruses were titrated (and were in most cases ca.  $10^6$  to  $10^7$  TCID<sub>50</sub>/ml), in some cases a  
338 4<sup>th</sup> passage in BHK cells was required to reach this titre.  
339 The rescued serotype O viruses O1K/O1M VP1 E83K and O1K/O1M VP1 K210E viruses have been  
340 described previously (Gullberg *et al.*, 2013b; Gullberg *et al.*, 2014).  
341  
342 *RT-PCR and Next Generation Sequencing (NGS).*  
343 For the purpose of NGS, extracted RNA (isolated as described above from virus harvests) was  
344 converted to cDNA using SuperScript III (Invitrogen) with a T<sub>27</sub> reverse primer according to the  
345 manufacturer's protocol. Two cDNA amplicons were prepared by PCR using Phusion DNA  
346 polymerase (Thermo Fisher) with the primers 13-N PN 2 + 10-P PN 30 (Supplementary Table S1)  
347 and separately 8-A PN 200 + 13-N PN 3 (Supplementary Table S1). These overlapping fragments  
348 (ca. 4kb and 4.2kb respectively) correspond to the most of the FMDV genome (downstream of the  
349 poly(C) tract, see Belsham (2005)), including the complete polyprotein coding region (ca. 7kb) but  
350 excluding the S-fragment at the 5'-terminus of the viral genome. The fragments were gel purified,  
351 mixed and then analysed by NGS essentially as described previously (Fahnøe *et al.*, 2014; Hadsbjerg  
352 *et al.*, 2016). The parental sequences of the FMDV chimeric O1K/O1M cDNA (as described by  
353 Gullberg *et al.*, 2013b) was assembled from the O1K sequence (Acc. No. X00871) and the coding  
354 sequence for the O1M capsid proteins (from Acc. No. AY593823) with known differences (see  
355 Gullberg *et al.*, 2013b), while the chimeric O1K/A22 sequence was generated using the O1K  
356 sequence and the A22 Iraq sequence (Carrillo *et al.*, 2005, Acc. No. AY593764.1). The derived  
357 sequences were used as the reference for mapping of sequence reads using Samtools (Li *et al.*, 2009),  
358 VarScan 2 (Koboldt *et al.*, 2012) and VCFtools (Danecek *et al.*, 2011), in succession, in order to  
359 generate consensus sequences from the mapped reads. Subsequently, consensus sequences were

360 aligned using MAFFT in Geneious (Biomatters, Auckland, New Zealand). Finally, a combination of  
361 Samtools (Li *et al.*, 2009), Lo-Freq (Wilm *et al.*, 2012) and SnpEff (Cingolani *et al.*, 2012) was used  
362 for downstream single nucleotide variant (SNV) analysis.

363

#### 364 *Virus infection of BHK cells.*

365 Virus titers were determined, as tissue culture infectious doses (TCID<sub>50</sub>), by titration in BHK cells  
366 according to standard procedures (Reed & Muench, 1938).

367 Monolayers of BHK cells, grown in 35-mm wells, were inoculated with the rescued viruses at a  
368 multiplicity of infection (MOI) of 0.1 TCID<sub>50</sub>/cell. At the indicated times post infection, cell lysates  
369 were prepared using 20 mM Tris-HCl (pH 8.0), 125 mM NaCl and 0.5% NP-40, and clarified by  
370 centrifugation at 18,000 × g for 10 min at 4°C.

371

#### 372 *Immunoblot analysis.*

373 Immunoblotting was performed, using cell lysates, according to standard methods as described  
374 previously (Polacek *et al.*, 2013). Briefly, aliquots of cell lysate were mixed with Laemmli sample  
375 buffer (with 25mM dithiothreitol), the proteins were separated by SDS-PAGE (12.5% or 4-15%  
376 polyacrylamide) and transferred to polyvinylidene difluoride membranes (PVDF, Millipore). Specific  
377 proteins were detected with primary antibodies recognizing: FMDV VP2 (monoclonal antibody 4B2,  
378 a gift from L. Yu, as described by Yu *et al.*, 2011) and FMDV 2A (ABS31; Millipore). Bound proteins  
379 were visualized using appropriate horseradish peroxidase-conjugated secondary antibodies (Dako)  
380 and a chemiluminescence detection kit (ECL Prime, Amersham) with a Chemi-Doc XRS system  
381 (Bio-Rad).

382

#### 383 *Immunofluorescence assays.*

384 Monolayers of BHK cells, grown on glass coverslips in 35-mm well plates, were infected with the  
385 rescued O1K/A22 viruses (MOI 0.1). At 6-8 hrs post infection, the cells were fixed, stained and  
386 mounted as previously described (Gullberg *et al.*, 2013b) using rabbit anti-FMDV A-Iraq serum and  
387 anti-FMDV 2A (ABS31, as above) followed by a donkey Alexa-fluor 568-labelled anti-rabbit IgG  
388 (A10042, Life Technologies). The slides were mounted with Vectashield (VECTOR laboratories)  
389 containing 4',6-diamidino-2-phenylindole (DAPI) and images were captured using an  
390 epifluorescence microscope.

391

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396

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529

530     **TABLES**

531     **Table 1. Analysis of SNVs within rescued O1K/O1M viruses as determined by NGS.**

Nt position	wt	Variant	O1K/O1M VP1 E83K	O1K/O1M VP1 K210E	O1K/O1M VP1 K210E	SNV effect (whole polyprotein)	SNV effect (individual protein)
			(Psg 2) (%) <sup>1</sup>	(Psg 2) (%) <sup>1</sup>	(Psg 3) (%) <sup>1</sup>		
762	T	C	6	-	-	(in 5' UTR)	-
948	T	G	-	1	3	(in 5' UTR)	-
1811	T	C	6	-	-	-	-
1875	A	G	3	-	-	T253A	VP4 (T52A)
2102	C	T	3	-	-	-	-
2357	A	G	-	3	3	-	-
3537	G	A	100	78	99	E807K	VP1 (E83K)
3918	A	G	-	100	100	K934E	VP1 (K210E)
3920	A	C	4	-	-	K934N	VP1 (K210N)
3920	A	T	9	-	-	K934N	VP1 (K210N)
3928	T	C	83	-	-	L937P	2A (L2P)
5166	A	G	3	-	-	S1350G	2C (S243G)
7392	T	C	-	5	7	F2092L	3D (F228L)
7765	A	G	2	-	-	K2216R	3D (K353R)

532

533     1: The percentage of each variant ( $\geq 1\%$ ) is given to the nearest integer.

534

535 **Table 2. Analysis of SNVs within rescued O1K/A22 viruses as determined by NGS.**

Nt position	wt	Variant	O1K/A22	O1K/A22	O1K/A22 VP1	SNV effect (whole polyprotein)	SNV effect (individual protein)
			(wt)	2A L2P	K210E 2A L2P		
			(Psg 2)	(Psg 3)	(Psg 3)		
			(%) <sup>1</sup>	(%) <sup>1</sup>	(%) <sup>1</sup>		
932	A	C	-	2	-	(in 5'-UTR)	-
1060	T	G	2	-	-	(in 5'-UTR)	-
1124	T	A	-	7	-	N2K	L (N2K)
1341	C	T	-	-	4	P75S	L (P75S)
1400	G	T	-	-	2	-	-
1865	C	T	-	2	-	-	-
2175	T	C	-	2	-	F353L	VP2 (F67L)
2342	A	G	-	-	2	-	-
2684	C	T	-	3	-	-	-
3154	T	C	-	1	-	V679A	VP3 (V174A)
3824	G	C	2	-	-	-	-
3884	T	C	-	8	-	-	-
3921	A	G	-	-	100	K935E	VP1 (K210E)
3930	T	C	-	90	100	L938P <sup>2</sup>	2A (L2P) <sup>2</sup>
3931	T	C	-	98	100		
3932	G	A	-	99	100		
4572	A	G	-	13	-	T1152A	2C (T44A)
4660	C	T	-	-	100	A1181V	2C (A73V)
4821	A	C	-	1	-	I1235L	2C (I127L)
4843	G	T	-	1	-	R1242I	2C (R134I)
5080	C	T	-	5	-	T1321I	2C (T213I)
6924	C	T	-	11	-	-	-
7170	T	C	3	-	-	Y2018H	3D (Y155H)
7358	C	T	-	-	4	-	-

536

537 1: The percentage of each variant ( $\geq 1\%$ ) is given to the nearest integer.

538 2: Change of the TTG to CCA codon was introduced by site-directed mutagenesis and the 3

539 changes together result in the 2A (L2P) substitution.

540

541

542 **Table 3. Influence of residue K210 in VP1 on VP1/2A junction cleavage in FMDV-infected**  
543 **cells.**

Virus	VP1 210 codon	Viability	Rescued virus sequence	Rescued virus amino acid	Comment	VP1/2A cleavage <sup>1</sup>
pO1K/A22 K210	AAA	+	AAA	K	wt	+
pO1K/A22 K210Q	<b>CAA</b> <sup>2</sup>	+	CAA	Q		-
pO1K/A22 K210R	<b>AGA</b> <sup>2</sup>	+	AGA	R		+
pO1K/A22 K210A(v1) <sup>3</sup>	<b>GCA</b> <sup>2</sup>	+	GCA	A		-
pO1K/A22 K210A(v2) <sup>3</sup>	<b>GCG</b> <sup>2</sup>	+	GCG	A		-
pO1K/A22 K210V	<b>GTT</b> <sup>2</sup>	+	GTT	V		-
pO1K/A22 K210M	<b>ATG</b> <sup>2</sup>	+	ATG	M		-
pO1K/A22 K210N	<b>AAC</b> <sup>2</sup>	+	AAC	N		-
pO1K/A22 K210E(v1) <sup>3</sup>	<b>GAA</b> <sup>2</sup>	+	AAA	K	Reversion	+
pO1K/A22 K210E(v2) <sup>3</sup>	<b>GAG</b> <sup>2</sup>	+	GAG	E		-

544

545 1: VP1/2A cleavage was assessed from IF staining using anti-2A antibodies (as in Fig. 4) and by  
546 immunoblotting (Fig. 5(a)).

547 2: nt changes in this codon are indicated in bold font.

548 3: (v1 or v2 to distinguish different codons)

549

550 **Figure legends**

551

552 **Figure 1. Schematic structure of the plasmid containing the FMDV O1K/A22 cDNA and**  
553 **derivatives.** The *NheI* and *ApaI* restriction enzyme sites (as indicated) were used as described in  
554 Materials and Methods to introduce cDNA fragments encoding the serotype A FMDV capsid proteins  
555 VP2-VP3-VP1-2A (from A22 Iraq, white fill) into the plasmid pT7S3 (Ellard *et al.*, 1999), containing  
556 a full-length cDNA corresponding to the O1Kaufbeuren B64 strain of FMDV (coding sequences  
557 marked in grey). The plasmid encoded amino acid sequences at the VP1/2A junction are shown. The  
558 FMDV O1K/A22 wild-type, single mutants (VP1 K210E or 2A L2P) and double mutant (VP1 K210E  
559 and 2A L2P) were produced as described in Materials and Methods. The full-length plasmids were  
560 linearized using *HpaI* prior to *in vitro* transcription and virus rescue. The locations of restriction sites  
561 used are marked: *NheI*, *ApaI* and *HpaI*. Sequence changes in the capsid coding region of the rescued  
562 viruses are indicated.

563

564 **Figure 2. Detection of FMDV capsid proteins in BHK cells infected with O1K/A22 wild-type**  
565 **and mutant viruses.** BHK cells were infected with O1K/A22 wild-type or mutant viruses (single  
566 mutant 2A L2P or double mutant VP1 K210E and 2A L2P) (MOI of 0.1) and whole cell lysates were  
567 prepared at the indicated times post infection. The presence of FMDV VP2 (and its precursor VP0)  
568 were detected by immunoblotting using anti-VP2 antibodies (panel (a)) and FMDV 2A (attached to  
569 VP1 as VP1-2A) was detected using anti-2A antibodies (panel (b)). Uninfected BHK cells were used  
570 as a negative control. Molecular mass markers (kDa) are indicated on the left.

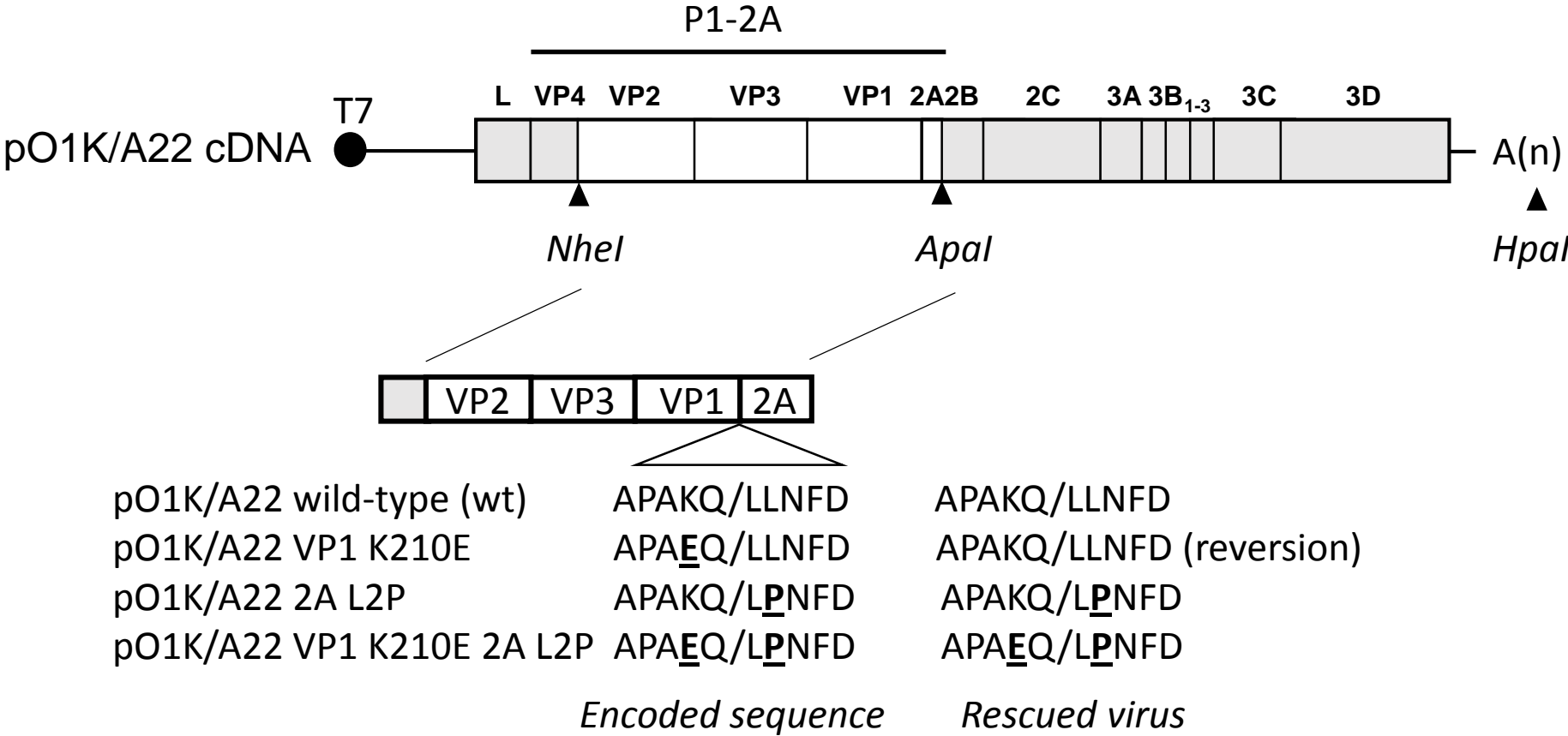
571

572 **Figure 3. Immunofluorescence staining of FMDV proteins within serotype A FMD virus-**  
573 **infected cells.** FMDV proteins within uninfected or FMDV-infected cells (using MOI of 0.1) were  
574 detected (at 8 h p.i.) using an anti-FMDV A-Iraq polyclonal antibody (panels (a) - (d)) or an anti-2A

antibody (panels (e) - (h)) and a secondary antibody labeled with Alexa Fluor 568 (red). Uninfected cells are shown in panels (a) and (e). Cells were infected with the viruses O1K/A22 (wild-type) (panels (b) and (f)), O1K/A22 2A L2P (panels (c) and (g)) or O1K/A22 VP1 K210E and 2A L2P (panels (d) and (h)) as indicated. The cellular nuclei were visualized with DAPI (blue). Bar, 50  $\mu$ m.

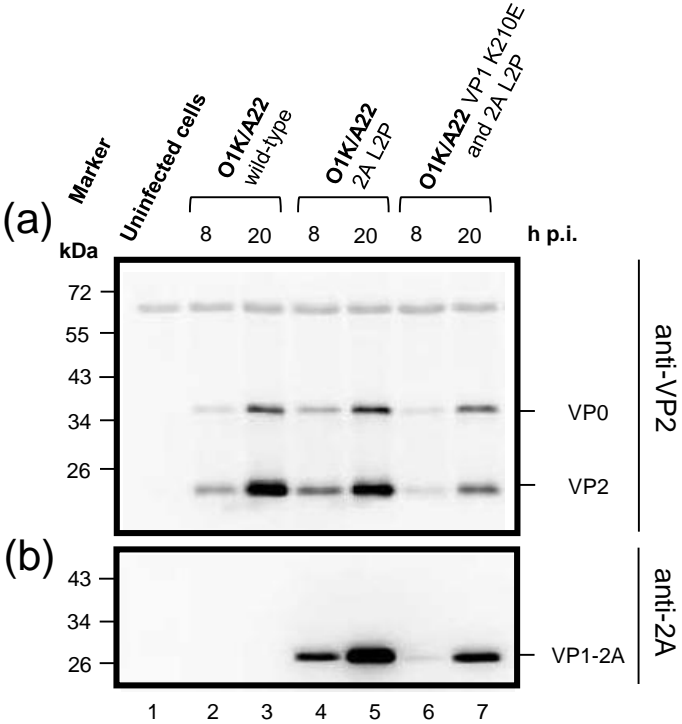
**Figure 4. Determination of VP1/2A cleavage by immunofluorescence staining for FMDV proteins within cells.** FMDV proteins within uninfected or FMDV-infected cells (using MOI of 0.1) were detected (at 6 h p.i.) using an anti-FMDV A-Iraq polyclonal antibody or an anti-2A antibody (as indicated) and a secondary antibody labeled with Alexa Fluor 568 (red) as in Fig. 3. The codon for residue 210 within VP1 (in parentheses) and the resulting individual amino acid residue within the different rescued viruses are indicated. Uninfected cells were used as a negative control. The cellular nuclei were visualized with DAPI (blue). Bar, 200  $\mu$ m.

**Figure 5. Assessment of FMDV VP1/2A cleavage in FMDV-infected BHK cells by immunoblotting.** Uninfected or FMDV-infected BHK cell lysates were analysed by SDS-PAGE and immunoblotting (as in Fig. 2). Where applicable, the cells were infected with the indicated viruses (at an MOI of 0.1) and the presence of FMDV 2A (attached to VP1 as VP1-2A) was detected using anti-2A antibodies (panel (a)) while FMDV VP2 (and its precursor VP0) were detected by immunoblotting using anti-VP2 antibodies (panel (b)). Uninfected cells were used as a negative control. Molecular mass markers (kDa) are indicated on the left.



## Figure 2

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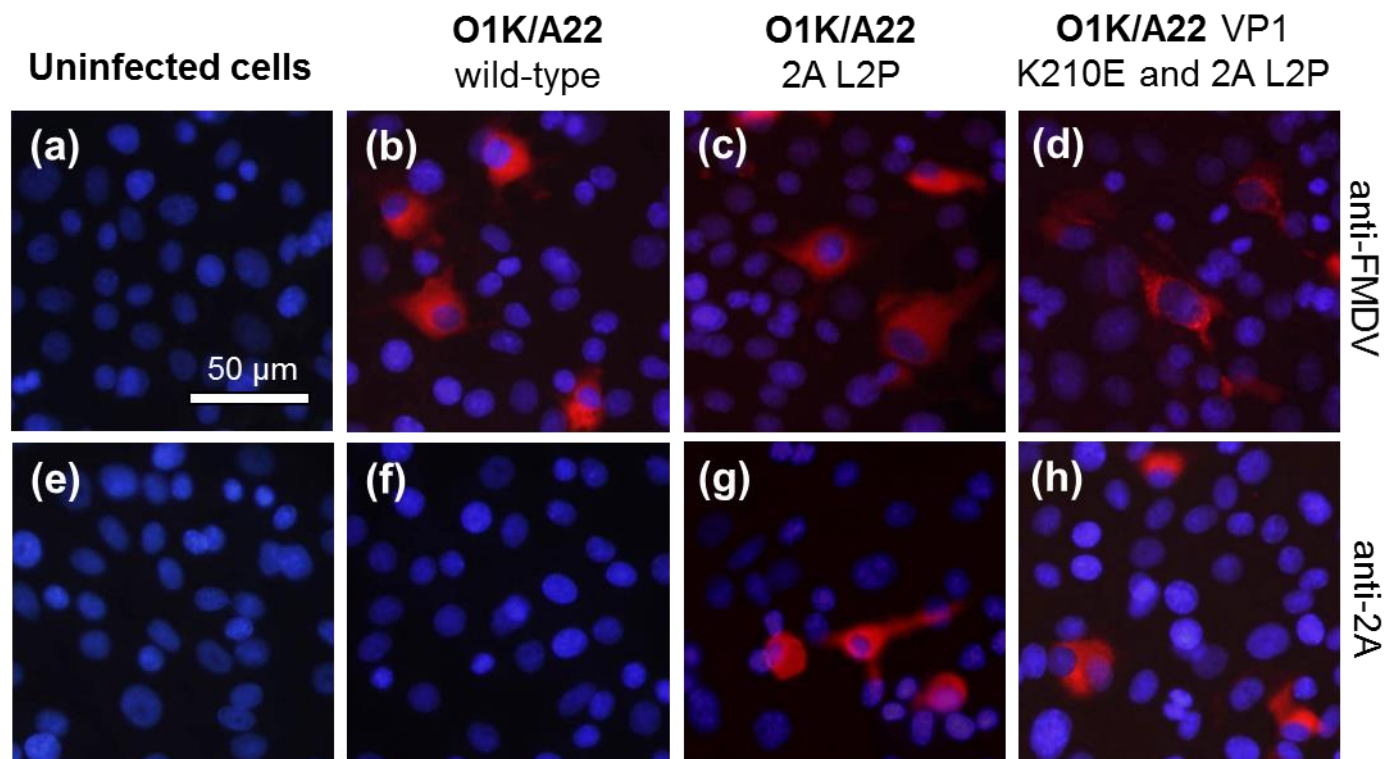


Fig. 3

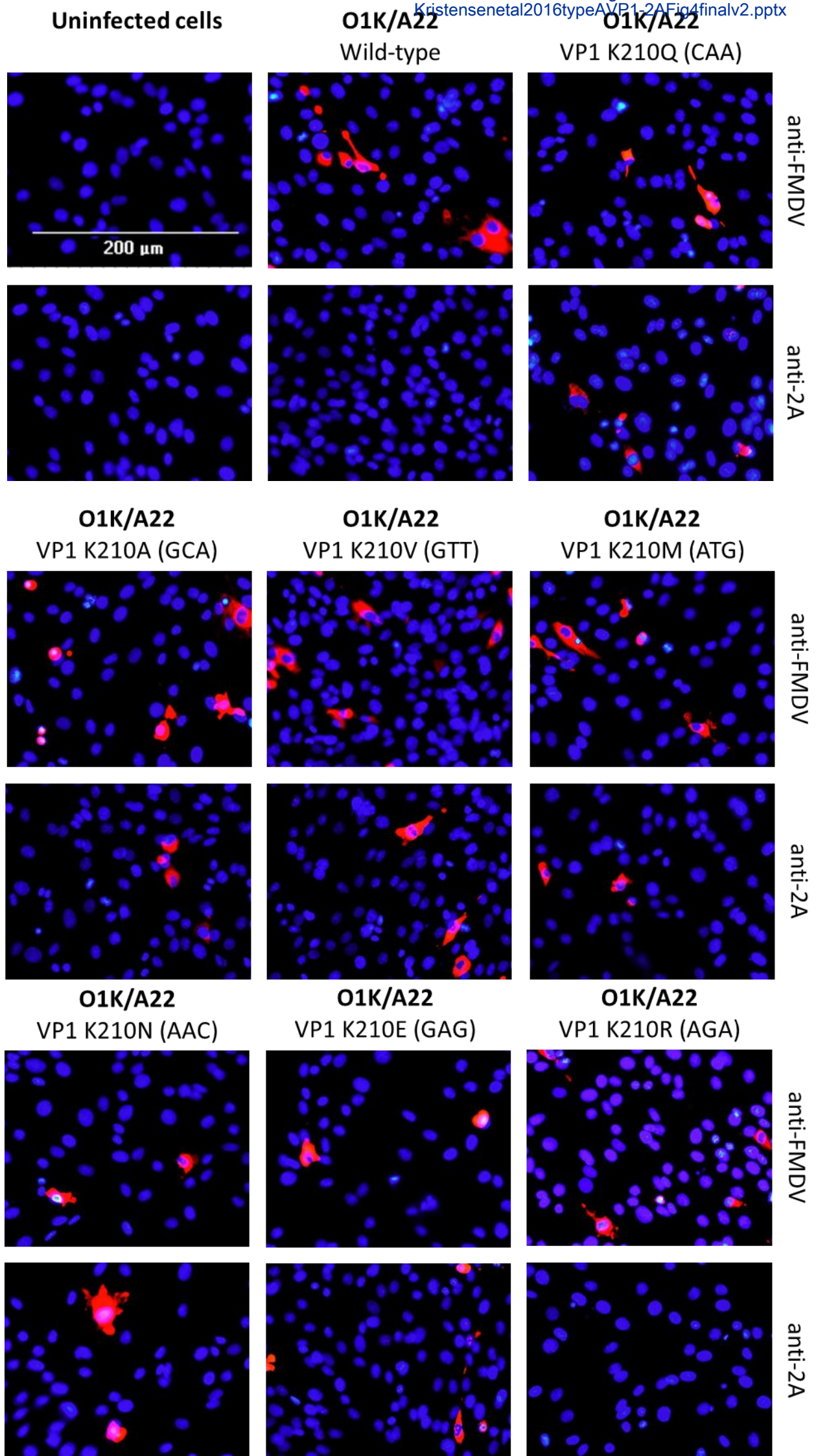


Figure 4

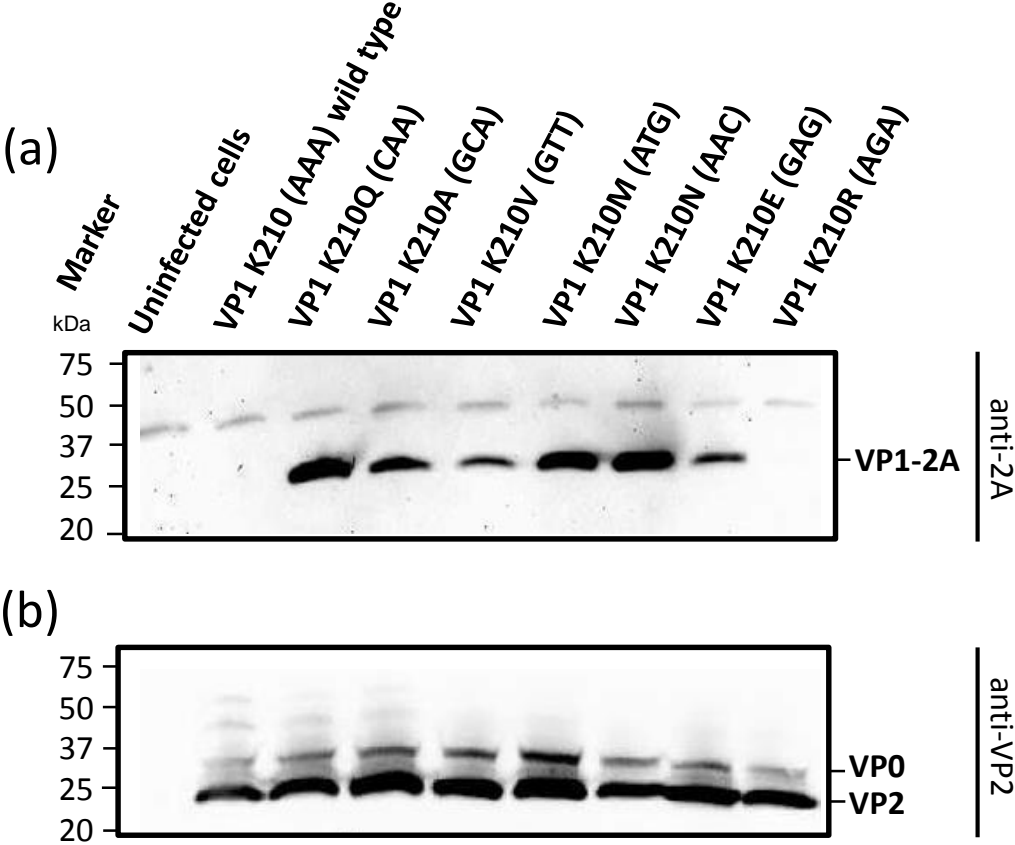


Fig. 5

**Supplementary Table S1. Primers used for plasmid construction and cDNA synthesis**

Primer name	Sequence (5'-3')
FMDVA_ <i>Nhe</i> IVP4VP2_Fw	CGCTCT <u>GCTAGCC</u> GATAAGAAGACCGAGGAGACCA
FMDVA_ <i>Apa</i> I2A2B_Re	CTACTAG <u>GGGCCC</u> GGGGTTGGACTCAACGTCTCCTG
FMDVA_2AL2P_Fw	CAACTT <b>CC</b> AACTTCGATTGCTCAAGTTGGCAGGAGAC
FMDVA_2AL2P_Re	GAAGTT <b>TGGA</b> AGTTGTTTTGCAGGTGCAATGATCTTCTG
FMDVA_VP1K210E_2AL2P_Re	GAAGTT <b>TGGA</b> AGTTGTTCTGCAGGTGCAATGATCTTCTG
13-N PN2	AAGTTTTACCGTCGTTCCCGACGTAAAAGGGAGGTAACCAC AAGCTTGAA
10-P PN 30	TCTGGACAGCACCTTTGTCTG
8-A PN 200	GAGACGTTGAGTCCAACCC
13-N PN 3	CCGTAGGAGTGAAAATCCCGAAAGGGTTTTTCCCGCTTCCTT AATCCAAA
O1PN20	GACATGTCCTCCTGCATCTG
13LPN21	GCACCTGCAN <b>NN</b> CAACTTTTGAAC

<sup>a</sup> Underlined sequences represent restriction enzyme sites *Nhe*I and *Apa*I. Nucleotide changes producing amino acids substitutions are shown in boldface italics.